Amaranthus seed regeneration in plastic tents in greenhouses

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Summary

Amaranthus germplasm is efficiently regenerated in plastic tents within greenhouses. This protocol involves the cultivation of 100-plant populations in 0.8 m^2 x 1 m tall tents, and generally yields 40 000-120 000 seeds about 3 months after planting. Experiments were performed to test the effectiveness of pollination control by isolating four monoecious *Amaranthus* species in these plastic tents. The estimated frequency of pollen contamination between tents was 0.01%. The estimated frequency of cross-pollination within tents was 3.9%.

Introduction

Amaranthus species are cultivated as a nutritious pseudocereal (Williams and Brenner 1995; Lehmann 1996) and as a leafy vegetable. Approximately 2000-3000 ha are grown annually in the United States for grain that is incorporated into health foods (Stallknecht and Schulz-Schaeffer 1993), but the crop is far more important in other parts of the world (Yue *et al.* 1993; Vinning 1995).

The *Amaranthus* germplasm collection of the United States Department of Agriculture's National Plant Germplasm System is located at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa. The collection contains about 3300 accessions including cultivated grain and vegetable types and wild species from many parts of the world.

Most of the accessions originated from seed samples that arrived at the NCRPIS in such small quantities that regeneration was required before the seeds could be distributed. Since the mid-1980s, 100-300 regenerations have been performed annually, mostly involving these new accessions.

In the climate of central Iowa, a greenhouse is the safest place to grow amaranths for seed regeneration because many germplasm accessions are unable to mature seeds under field conditions. When the plants are cultivated under short photoperiods in the greenhouse, seeds are generally harvested about 2-4 months after planting. This relatively rapid cycling of crops enables efficient use of limited greenhouse space.

Pollination control

Germplasm curators have recognized at least since 1977 that *Amaranthus* germplasm should be regenerated with controlled pollination (Kauffman 1979). The mean outcrossing rates under field conditions have been variously estimated at 3.5-34% (Jain *et al.* 1982; Hauptli and Jain 1985; Agong and Ayiecho 1991) for those species grown as pseudocereals. Insects may be important pollinators and could account for some variability in outcrossing rates (Hauptli and Jain 1985). Within the genus *Amaranthus*, outcrossing rates have not been measured for most of the approximately 60 species, but some *Amaranthus* species are dioecious and, therefore, obligately outcrossing (Sauer 1957).

Three methods of pollination control are available for *Amaranthus* germplasm regeneration: physical isolation, bagging the heads of individual plants, and enclosing groups of plants in tents. The isolation method requires large field areas or greenhouse space and has not been evaluated rigorously. The bagged-head method requires fastening paper bags over the developing inflorescences, which then self pollinate. At the NCRPIS, this has been used for controlled pollination of locally adapted germplasm in field plantings. Large white pollination bags manufactured for use on *Zea* are suitable for *Amaranthus*. We use Lawson #421 bags from the Lawson Company* of Northfield, Illinois.

The use of plastic tents within a greenhouse has become the primary regeneration method at the NCRPIS because it is suited to germplasm that is poorly adapted to our local field conditions. It also has the advantage of allowing for some outcrossing among plants of the same accession, which is desirable for maintaining inherent levels of heterozygosity.

The Rodale Research Institute amaranth programme location used plastic partitions to separate accessions during greenhouse regeneration (Weber and Kauffman 1990). Similarly, at the NCRPIS, M. Millard used plastic tents for seed regeneration beginning in 1982; these tents are described by Lehmann *et al.* (1991) and also briefly described by Williams and Brenner (1995). The following paragraphs describe the most recent refinement of Lehmann's and Millard's earlier methods.

Clear plastic tents, supported by plastic frames, are designed to prevent pollen flow between accessions. These tents are 152 x 91 x 71 cm, made of 0.0038-cm (1.5-mil) polyethylene, purchased from the Associated Bag Company*, Milwaukee, Wisconsin. The tents reach 1 m above

the soil level and are ventilated with two horizontal 15-cm cuts near their tops. The tent frames are assembled from standard 2.1-cm diameter polyvinyl chloride pipes and fittings purchased locally.

The tents are placed over the plants when the inflorescences begin to emerge. The edges of the tents are secured with clothespins to flats in which the plants are rooted. Plants inside the tents have reduced need for watering because evapotranspiration is reduced. Watering is facilitated with irrigation hoses positioned on top of the flats (Fig. 1).

Regeneration protocol

Each accession is grown in three side-by-side plastic flats (Fig. 1), each 54 cm x 28 cm x 6 cm, containing about 6 L of a growing medium of 20% soil, 40% sphagnum peat, and 40% perlite. The flats have no partitions, and together hold approximately 100 plants. The plants grow to seed maturity when 40 to 100 cm tall, in 0.8 m^2 (6 ft^2), yielding approximately 40 000-120 000 seeds (20-60 g).

Seed dormancy sometimes complicates stand establishment, especially of wild species. The physiology of *Amaranthus* seed dormancy was reviewed by Kigel (1994). In most cases, dormancy can be overcome with cool-moist seed treatments. The seeds are placed on moist blotter paper in plastic boxes at 2-5°C for at least a month, then the boxes are moved to a 20/30°C growth chamber, where most seeds germinate within 2 weeks. Shortly after germination, the seedlings are transplanted into the growing medium. Germinating in boxes enables dead seeds to be distinguished from dormant seeds. The domesticated (especially white-seeded) accessions generally exhibit no seed dormancy and are planted directly in the growing medium at a depth of approximately 0.25-0.5 cm. Seedlings of the domesticated accessions emerge less than 1 week after planting when cultivated at approximately 20-35°C.

Fig. 1. Greenhouse *Amaranthus* seed production with plastic tents to control pollination. The lower edges of the tents are secured with clothespins. Bolts fastened to the benches keep the tent frames in position. The black hoses on the flats emit irrigation water.

Most accessions in the NCRPIS germplasm collection are daylength sensitive and will flower and produce seeds in less than 3 months if cultivated under photoperiods shorter than 12 hours. Short photoperiods also reduce overall plant size and crowding. This is also valuable for keeping plants small. Photoperiod manipulation was used by Lehmann (1995) for "rapid cycling" of amaranths. *Amaranthus cruentus* L. accessions are the least sensitive to daylength, and thus have long periods of vegetative growth (Weber and Kauffman 1990). In contrast, some temperate wild accessions are so photoperiodic that they must be started in long days before exposure to short days, or they will flower when too small and yield poorly.

Curtains limit the photoperiod to 12 hours or less within a greenhouse, or regeneration occurs in the winter, when the natural photoperiod is short. If the short-day treatment is discontinued, flowering will cease and vegetative growth will resume. Some short-day plants will eventually flower under long-day conditions in a greenhouse after four or more months of growth. Zabka (1961) and Kigel (1994) reported additional information about photoperiod effects on amaranths.

The second method of reducing vegetative growth is to cultivate seedlings in a medium with low levels of nutrients. The young plants are not fertilized, and nutrient levels in the medium are low enough to reduce growth. Older plants are fertilized heavily only after infloresences emerge, so that the plants are vigorous during flowering and seed maturity. Our primary fertilizer is a pelletized slow-release 17-6-12 (NPK) from the Grace Sierra Horticultural Products Company* of Milpitas, California.

A third method to reduce plant size is to prune tall plants to about 35 cm after they have grown to the top of the pollination cage. Unfortunately, this can postpone flowering, but it is useful for regenerating some photoperiod-insensitive accessions.

A final method for reducing plant size is manipulation of day and night temperatures, as routinely used in the floriculture industry (Nelson 1991:384-390), but this has not been evaluated rigorously for *Amaranthus*.

Experimental tests of pollination control

The four amaranth species evaluated here (Table 1) are the most economically important for grain and vegetable pro-

Table 1. Plant materials used in the experiments

Species	Seedling pigmentation[†]	Accession	Other Identifiers
A. caudatus	green	PI 511679	HH 4, LSK 369, RRC 551
	red	PI 553073	Love Lies Bleeding, RRC 306
A. cruentus	green	PI 482049	TGR 542
	red	PI 477914	R 149, RRC 1041
A. hypochondriacus	green	PI 477915	R 159, RRC 1008
	red	PI 477917	R 103, RRC 1024
A. tricolor	green	PI 527321	Bai Lui Ye Xian
	red	Ames 5147	Lalsag Bhaji, RRC 389

[†]Referred to in the text as "green accessions" or "red accessions".

duction. In some instances, these same accessions have been used for pollination studies by other authors (Agong and Ayiecho 1991; Lehmann *et al.* 1991). Additional information about these and other accessions is available via the internet at<http://www.ars-grin.gov>. This internet site provides public access to the Germplasm Resources Information Network (GRIN) computer database of the USDA-ARS National Plant Germplasm System.

A dominant allele for red pigmentation in seedlings was used as a genetic marker for cross-pollination. Kulakow *et al.* (1985) studied this trait and used the notation R/r to describe its simple monogenic inheritance, with red (R) dominant to green (r). The few seedlings with off-type colors were culled from the plantings soon after germination. Early emerging inflorescences were removed to synchronize the flowering of cross-compatible accessions and to encourage outcrossing. Seeds were harvested from the experiments for testing the extent of (1) mutation at the R locus, (2) cross-pollination within tents, and (3) cross-pollination between tents. Seedlings were evaluated for colour within 14 days of germination on moist blotter paper in plastic boxes at 20/30°C.

Measurement of mutation rate at the R locus

Green plants of all four accessions (Table 1) were cultivated under complete isolation during the winter of 1993/94 in a greenhouse that contained no red *Amaranthus* plants. No wild plants grow outdoors in Iowa during this season, eliminating the possibility of pollen contamination via the greenhouse ventilation system. Seeds were harvested and germinated to count the frequency of red seedlings, which would indicate mutation to the dominant red allele.

In these tests, not one copy of the green allele mutated to the red allele (Table 2), indicating a likely mutation rate lower than 0.01%. By comparison, Behera and Patnaik (1979) reported a combined mutation rate lower than 0.08% for six inflorescence structural mutations in *Amaranthus*. These data indicate that mutations are a very small potential source of error for our pollination-control experiments.

Measurement of pollen movement within tents

Individual green plants were surrounded with a cross-compatible "red accession" within three plastic tents. At the time of flowering, each flat contained approximately 45 red plants and one green plant. The red plants were pruned so they would not excessively shade the green plant. Seeds were harvested from green plants and germinated to count the frequency of red seedlings, which resulted from pollen movement within the plastic tents (Table 3).

Table 2. Frequency of mutation from the green to the dominant red allele

Species	Red seedlings	Green seedlings
A. caudatus	0	3833
A. cruentus	0	1502
A. hypochondriacus	0	3724
A. tricolor	0	1483
Total	0	10524

The frequency of outcrossing could be up to twice as great as these data indicate because the "red accessions" may include heterozygous plants. But few off-type green plants were culled from the "red accessions" early in the experiment. Therefore these data should provide a useful estimate of the relative rates of outcrossing.

Table 3. Frequency of cross-pollination within plastic tents in a greenhouse

Species	Red seedlings	Green seedlings	% Red seedlings
A. caudatus	75	1540	4.9
A. cruentus	35	710	4.9
A. hypochondriacus	13	560	2.3
A. tricolor	49	1619	3.0
Total	172	4429	3.9

Measurement of pollen flow between tents

Tents containing a "green accession" were surrounded with four tents containing a synchronously flowering cross-compatible "red accession". Seeds were harvested from the "green accessions" and germinated to count the frequency of red seedlings, which should reflect pollen contamination rates among tents.

Contamination was detected only once in 10 245 seedlings (0.01%) (Table 4). Even accounting for the possibility that the "red accessions" may be heterozygous, this frequency of contamination is so low that it may fall within the range of mutation rates.

Table 4. Frequency of pollen contamination among plastic tents in a greenhouse

Species	Red seedlings	Green seedlings
A. caudatus	1	3521
A. cruentus	0	1365
A. hypochondriacus	0	3652
A. tricolor	0	1707
Total	1	10245

Discussion

The *Amaranthus* seed regeneration method described herein is effective and contributes to the managerial goals set for the National Plant Germplasm System (Clark *et al.* 1997). Pollen flow between tents is at an acceptably low level, whereas pollen flow within populations occurs at a rate that may approximate that in natural populations. These regeneration methods may also be appropriate for other genera, such as *Chenopodium* and *Spergula*, that do not require insects for pollination and that tolerate high-density cultivation. We are conducting trials to determine which other genera may be regenerated successfully with this system.

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*Mention of commercial brandname products does not constitute an endorsement of any product by the US Dept. of Agriculture, Agriculture Research Service (USDA-ARS) or cooperating agencies.

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Résumé

*Régénération de semences d'*Amaranthus *sous tentes de plastique en serre*

Le germoplasme d'*Amaranthus* est régénéré avec efficacité sous tentes de plastique en serre. Ce protocole comprend la culture de populations de 100 plantes sous tentes d'une surface de 0.8 m^2 et d'une hauteur d'1 m, et produit généralement 40.000 à 120.000 semences environ 3 mois après le semis. Des essais ont été réalisés pour tester l'efficacité du contrôle de la pollinisation en isolant quatre espèces monoïques d'*Amaranthus* dans ces tentes de plastique. La fréquence estimée de contamination du pollen entre tentes était de 0,01%. La fréquence estimée de pollinisation croisée à l'intérieur d'une tente était de 3,9%.

Resumen

Regeneración de semillas de Amaranthus *en tiendas de plástico en invernaderos*

El germoplasma de *Amaranthus* se regenera de manera eficiente en tiendas de plástico dentro de invernaderos. Este protocolo supone el cultivo de poblaciones de 100 plantas en tiendas de 0.8 m^2 y de 1m de altura, y generalmente produce entre 40.000 y 120.000 semillas tres meses después de la siembra. Cuatro especies monoecias de *Amaranthus* fueron aisladas a fin de evaluar la eficacia del control de la polinización en estas tiendas de plástico. La frecuencia aproximada de la contaminación del polen en las tiendas fue del 0.01%. La frecuencia aproximada de polinización cruzada fue del 3.9%.